Sevoflurane preconditioning protects from posttransplant injury in mouse lung transplantation

Yamada, Yoshito ; Laube, Isabelle ; Jang, Jae-Hwi ; Bonvini, John M ; Inci, Ilhan ; Weder, Walter ; Beck-Schimmer, Beatrice ; Jungraithmayr, Wolfgang

Abstract: BACKGROUND: Although sevoflurane (Sevo) had been shown to ameliorate posttransplant injury in various organs, data available are inconsistent, particularly in the context of lung transplantation (Tx). We here investigated if preconditioning by Sevo can protect from posttransplant injury regarding both, primary graft dysfunction (PGD) and acute rejection (AR) after experimental lung Tx, thereby focusing on two important clinical outcome parameters. MATERIALS AND METHODS: Three experimental approaches were used: (1) BALB/c mice were preconditioned for 2 h with Sevo or a fentanyl cocktail (Control; n = 10); (2) syngeneic (Syn) mouse lung Tx (C57BL/6) with a Sevo-preconditioned graft followed by 18 h storage to mimic PGD (Syn-Tx, n = 12) versus controls (fentanyl cocktail); and (3) allogeneic (Allo) Tx (BALB/c, donor; C57BL/6, recipient) to mimic AR (Allo-Tx, n = 12) versus controls (fentanyl cocktail). Syn-Tx grafts were harvested on Day 1, Allo-Tx grafts on Day 3 and analyzed for histology, immunohistochemistry, blood gas analysis, and inflammatory cytokines (enzyme-linked immunosorbent assay or reverse transcription polymerase chain reaction). RESULTS: Evaluating the preconditioning effect of Sevo only showed significantly better oxygenation ($P = 0.03$) and a tendency toward lower levels of lung tissue messenger RNA for tumor necrosis factor- . In Syn-Tx recipients, the Sevo group had histologically a tendency toward an attenuation of PGD and showed significantly lower levels of interleukin 6 ($P = 0.01$) in plasma, but higher levels of interleukin 10 ($P < 0.01$) in lungs. Allo-Tx grafts in Sevo Tx recipients showed attenuated AR with histologically significantly lower rejection scores ($P = 0.03$), fewer classical macrophages (F4/80+; $P < 0.01$), but more anti-inflammatory activated macrophages (M2, CD206+; $P < 0.01$). Functionally, the Sevo group had a tendency toward improved oxygenation. CONCLUSIONS: We demonstrated that Sevo preconditioning has protective effects on lung transplants in both, PGD and AR. The observed amelioration may be attributed to suppressed inflammatory cytokines during PGD and the induction of alternatively activated macrophages during AR. These promising data could set the base for using Sevo preconditioning in donor lungs for a human trial.

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Sevoflurane preconditioning protects from post-transplant injury in mouse lung transplantation

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YY performed mouse lung transplantations (Tx), data analysis, and co-wrote the manuscript; IL participated in mouse lung Tx, data analysis and co-wrote the manuscript; JHJ assisted in Tx experiments and data analysis; JB, II, IY, WW and BBS co-drafted the manuscript (ms); WJ designed the experiments, interpreted the results and wrote the ms. All authors read and approved the final version of the ms.

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Abbreviations

Allo; allogeneic, AMPK; 5' adenosine monophosphate-activated protein kinase, AR; acute rejection, ATP; adenosine triphosphate, HE; hematoxylin and eosin, IHC; immunohistochemistry, I/R; ischemia reperfusion, PGD; primary graft dysfunction, RT-PCR; reverse transcription polymerase chain reaction, Sevo; sevoflurane, Syn; syngeneic, Tx; transplantation

Abstract

Background

Although sevoflurane (Sevo) had been shown to ameliorate post-transplant injury in various organs, data available are inconsistent, particularly in the context of lung transplantation (Tx). We here investigated if preconditioning by Sevo can protect from post-transplant injury regarding both, primary graft dysfunction (PGD) and acute rejection (AR) after experimental lung Tx, thereby focusing on two important clinical outcome parameters.

Materials and Methods

Three experimental approaches were employed: (I) BALB/c mice were preconditioned for 2 hours with Sevo or a fentanyl cocktail (Control) (n=10), (II) syngeneic mouse lung Tx (C57BL/6) with a Sevo-preconditioned graft followed by 18 hours storage to mimic PGD (Syn-Tx, n=12) vs. controls (fentanyl cocktail), and (III) allogeneic Tx (BALB/c, donor; C57BL/6, recipient) to mimic AR (Allo-Tx, n=12) vs. controls (fentanyl cocktail). Syn-Tx grafts were harvested on day 1, Allo-Tx grafts on day 3 and analyzed for histology, immunohistochemistry, blood gas analysis, and inflammatory cytokines (ELISA or RT-PCR).

Results

Evaluating the preconditioning effect of Sevo only showed significantly better oxygenation ($p=0.03$) and a tendency towards lower levels of lung tissue mRNA for TNF-α. In Syn-Tx receipients, the Sevo group had histologically a tendency towards an attenuation of PGD and showed significantly lower levels of IL-6 ($p=0.01$) in plasma, but higher levels of IL-10 ($p<0.01$) in lungs. Allo-Tx grafts in Sevo Tx recipients showed attenuated AR with histologically significantly lower rejection scores ($p=0.03$), fewer classical macrophages (F4/80+; $p<0.01$), but more anti-inflammatory activated macrophages (M2, CD206+; $p<0.01$). Functionally, the Sevo group had a tendency towards improved oxygenation.

Conclusions

We demonstrated that Sevo preconditioning has protective effects on lung transplants in both, PGD and AR. The observed amelioration may be attributed to suppressed inflammatory cytokines during PGD and the induction alternatively activated macrophages during AR. These promising data could set the base for employing Sevo preconditioning in donor lungs for a human trial.

Introduction

Lung transplantation (Tx) has been established for several decades as a therapeutic option for end-stage pulmonary disease patients. In spite of recent developments including surgery, immunosuppressive and perioperative management (1, 2), primary graft dysfunction (PGD) and acute rejection (AR) following lung Tx are still a major hurdle on the way to long term graft acceptance (3). PGD and AR are not only responsible for early transplant morbidity and mortality (4), these two pathologic entities contribute to the development of chronic lung allograft dysfunction, the major impediment to long-term survival of lung transplanted patients (5).

Preconditioning by sevoflurane (Sevo), a clinically frequently used volatile anesthetic, has been shown to attenuate I/R injury and also AR in several organs, such as liver, kidney, heart and lung (6-9) through reducing inflammatory cytokines (10, 11), chemokines (12) and adhesion molecules (13). A possible mechanism responsible for this beneficial effect has been attributed to the homeostasis of adenosine triphosphate (ATP) in mitochondria with slower calcium influx (14) and antioxidant activity (11, 13). Some data exist for Sevo preconditioning in lung Tx, but they are largely limited to the analysis of PGD, showing a decrease of the inflammatory response and oxidative stress of I/R injury (9, 15). Others demonstrated the efficacy of volatile anesthetics to attenuate I/R injury in an isolated lung model without Tx procedure (16, 17).

We therefore investigated here if Sevo preconditioning of the donor graft might have protective effects on both, PGD and AR. To this end, we applied different experimental set-ups to best mimic the human post-transplant setting by employing syngeneic mouse lung Tx for PGD, and allogeneic Tx for AR.

Materials and Methods

Mice

Specific pathogen-free inbred male mice, strain C57BL/6 (H2b) and BALB/c (H2d) (Charles River Laboratories, Sulzfeld, Germany), weighing 27–30 g were used. Animals received adequate care in strict accordance with the Principles of Laboratory Animal Care (National Institutes of Health Publication No. 85-23, promulgated in 1985, most recently revised in 1996). The study was approved by the local veterinary ethical committee under the study number 45/2014.

Experimental groups for the induction of PGD and AR

To investigate the impact of Sevo on donor preconditioning only, we performed preconditioning of Sevo (3% of Sevo for 2 hours) on the donor organ without Tx. In all other experiments, the orthotopic single left lung transplantation was performed as we described in detail previously (2, 18).

Two groups of Tx were formed: (I) syngeneic Tx (Syn-Tx, C57BL/6 →C57BL/6) and (II) allogeneic Tx (Allo-Tx, BALB/c → C57BL/6). Both groups, Syn-Tx as well as Allo-Tx were further subdivided into one donor group that received a Sevo preconditioning (3% of Sevo for 2 hours), and another donor group that received ip.-narcosis, in order to be able to discriminate the effect of Sevo preconditioning. All control mice received an ip.-narcosis by applying a volume of 2 µl/g of an anesthetic cocktail composed of 1 ml of 0.05% fentanyl, 5 mg of midazolam, 0.5 mg of medetomidin and 0.5 ml of 0.9% saline. The narcosis was maintained for 2 hours with 0.5 µl/g of 0.016% fentanyl administered at every 40 minutes after the induction and terminated by giving a cocktail of naloxon, flumazenil and atipamezol. All recipient mice received this type of ip.-narcosis without the use of Sevo. Syn-Tx mice underwent an 18 hour cold ischemia time prio to Tx, as we described before (19). Recipient mice in the Syn-Tx group were sacrifized at day 1, and in the Allo-Tx group at day 3 after Tx.

Graft harvest and assessment

Sacrifice of Tx recipient mice were performed as previously described (2, 18). 500µl of whole blood was aspirated from the inferior vena cava for assessment of cytokines, and 100µl of arterial blood was aspired from the descending aorta for blood gas analysis. Thereafter, lungs were flushed with 2 ml of 0.9% normal saline solution at a pressure of 10 cm H_2O via the pulmonary artery, and organs were subsequently removed. Harvested lungs were washed in PBS and snap frozen in liquid nitrogen and stored at -80 °C until performance of the assays.

Histology and pathological grading

Transplanted lungs from each group were harvested, fixed in 4% phosphate buffered formalin, cut and embedded in paraffin. Sections of 4 µm thickness were cut and stained for hematoxylin & eosin (HE). These sections were graded for rejection pathology using the standard criteria (20) and assessed by three different investigators in a blinded fashion.

Immunohistochemistry

Transplanted lungs were fixed in formalin (4%), paraffin embedded, and processed for immunohistochemistry (IHC). IHC staining was performed on BondMax with Refine HRP-Kit DS9800 (Leica Biosystems, Muttenz, Switzerland) according to the manufacturers' guidelines. Primary antibodies were rabbit anti-CD3 mAb (RMAB005, Diagnostic Biosystems, Pleasanton, USA), rat anti F4/80 mAb (T-1006, BMA Biomedicals, Augst, Switzerland), rat anti-CD45R (B220) (RA3-6B2, BD Pharmingen, Allschwil, Switzerland), rabbit anti-neutrophil elastase polyclonal antibody (ab21595, Abcam, Cambridge, UK) and rabbit anti-CD206 mAb (18704-1-AP, Proteintech, Chicago, USA). The number of positive cells in the perivascular and peribronchiolar areas was counted. Vessels or airways with the diameter of 150µm were the target for counting. Five sites on each section were chosen. Immuno-stained sections were assessed by three different investigators in a blinded fashion.

Oxygenation of lung transplants

PaO_2/FiO_2 ratio in the transplanted animal was taken from the abdominal aorta (100 µl) for blood gas analysis and electrolyte measurement test (Epocal Inc., Ottawa, ON Canada), in order to evaluate the function of the graft after lung Tx.

Quantitative real-time PCR

Total RNA was extracted by mirVana Paris kit (Ambion, Thermo Fisher Scientific, Waltham, MA) following the manufacturer's instructions. Five micrograms of RNA were used for reverse transcription by ThermoScript reverse transcription-PCR (RT-PCR) System (Invitrogen, Thermo Fisher Scientific) yielding cDNA template. Quantitative real-time PCR amplification and data analysis were performed using 7500 Fast Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific). Taq Man gene expression assays were used to quantify mRNA expression of the respective genes. mRNA expression levels of each sample were normalized to 18S RNA (Taq Man rRNA control reagents; Applied Biosystems). All mRNA expression data was calculated as fold change from naïve animal tissues.

Quantification of cytokines (ELISA)

Lung samples were homogenized in lysis buffer containing a protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany), phosphatase inhibitor cocktail 3, 50 mM Tris, 150 mM NaCl, 5 mM EDTA, and 0.5 % NP-40 (Sigma-Aldrich, St. Louis, USA). The concentration levels of IL-6, IL-10 and TNF-α in mouse plasma and lung homogenates were assayed by ELISA using the paired Abs for capture and detection according to the manufacturer's instructions (R&D Systems, Santa Cruz, USA).

Statistics

Data were presented as means ± standard deviation. Groups were compared with the Student t-test for unpaired samples using Prism 5 (GraphPad Software, San Diego, CA, USA). A two-sided p-value <0.05 was considered as statistically significant.

Results

Sevoflurane preconditioning improved oxygenation in donor lungs

In order to analyze the effect of Sevo on the donor organ only, we preconditioned a donor lung in animals with Sevo n=5) and without Sevo (control, n=5). PaO_2/FiO_2 ratio in the Sevo group was significantly higher than the control group (Fig. 1a) (p=0.030). RT-PCR of inflammatory cytokines showed a relative suppression of the production of TNF-α in the Sevo group (Fig 1b) (p=0.20).

Sevoflurane preconditioning attenuated PGD histologic injury in Syn-Tx.

Histologically, transplanted lungs in the control group (n=6) showed severe PGD features characterized by diffuse alveolar septal thickening with edema and neutrophilic infiltrates (Fig 2A) when compared to, the Sevo group which showed less severe damage of a PGD pathology (Fig 2B).

Sevoflurane reduced levels of IL-6 and enhanced levels of IL-10 in Syn-Tx

We performed ELISA to analyze levels of the pro-inflammatory cytokine IL-6 and the anti-inflammatory cytokine within recipient transplants. Levels of IL-6 in the Sevo group revealed significantly lower levels in plasma (Fig 3A) (p=0.012) and relatively lower levels in lung tissue (Fig 3B) (p=0.15) when compared to the control group. In contrast, levels of IL-10 in the Sevo group were significantly higher in lung tissue (Fig 3D) (p=0.002) and relatively higher in plasma (Fig 3C) (p=0.15).

Sevoflurane did not improve oxygenation in Syn-Tx

PaO_2/FiO_2 ratios in both groups on day 1 were comparable among animals (Control 389±99 versus Sevo 396±74; p=0.90).

Sevoflurane preconditioning attenuated AR in Allo-Tx.

Allogeneic orthotopic single-lung Tx was performed using a MHC class I and II fully mismatch combination strain between C57BL/6 and BALB/c mice in the Sevo (n=6) and the control group (n=6). Histology of control lung transplants, harvested on day 3 (n=6) showed severe AR pathology

characterized by perivascular and peribronchiolar mononuclear infiltration (Fig 4A). In contrast, the Sevo group had less infiltrates (Fig 4B). According to the rejection score (ISHLT guidelines, (20)), the Sevo group had a significantly reduced acute rejection score when compared to controls (Fig 4C) (p=0.030).

Sevoflurane preconditioning induced increased numbers of M2 macrophages in Allo-Tx

In order to identify a putative cell population that could play a protective role upon Sevo preconditioning, we analyzed for the anti-inflammatory subtype of macrophages, M2. To do so, we performed IHC staining using antibodies for F4/80+ (total macrophage), CD206+ (alternative activated M2 macrophage). Also, CD3+ (pan-T cell marker), B220+ (B cell marker) and Neutrophil elastase (neutrophil) were stained to further dissect cell populations in allo Tx recipients. Figure 5 shows representative IHC sections of F4/80+ and CD206+. F4/80+ cells were enhanced in controls compared to the Sevo group (Fig 5A, B). Also, the number of F4/80+ cells in the perivascular and peribronchiolar areas were significantly higher (Fig 5C) (p=0.001). The number of CD206+ macrophages was higher in the Sevo group (Fig 5F) (p=0.003) but there were less CD206+ macrophages in controls (Fig 5D, 5E). T cells, B cells and neutrophils, were not significantly different among animals.

Sevoflurane preconditioning improved oxygenation in Allo-Tx

There was a tendency of PaO_2/FiO_2 ratio improvement in the Sevo group when compared to controls though without significant difference (Fig. 6) (p=0.16).

Discussion

The efficacy of Sevoflurane preconditioning has been investigated in the context of solid organ transplantation (6, 7, 9). However, results obtained in clinical trials in liver and kidney transplantation turned out to be inconsistent (21, 22). With regard to lung Tx, there is virtually no data, neither on the impact of Sevo in lung transplant recipients nor on Sevo in donor organs. Our primary aim here was therefore to evaluate the impact of donor organ preconditioning on PGD and AR by Sevo. We found that Sevo preconditioning of the donor organ attenuates PGD and AR histology and improved oxygenation and upregulated protectiveM2 macrophages with a promotion of anti-inflammatory activity, therefore displaying protecting effects on lung Tx outcome.

During donor preconditioning, we could observe an improvement of oxygenation with a relative suppression of the inflammatory cytokine TNF-α. Indeed, our data were supported by previous reports about the efficacy of volatile anesthetics in a mouse model of ventilator-induced lung injury (11, 23). In this context, TNF-α was proved to be sensitive marker for acute lung injury (24). Only preconditioning of lung organs by Sevo without Tx showed a trend towards suppression of inflammation and thus gives a hint towards a beneficial effect of Sevo.

In order to stimulate and ensure robust I/R injury and thus PGD, we used the syngeneic Tx setting employing 18 hours ischemia. Here, a strong impact by Sevo preconditioning could be observed reflected by an improvement of PGD histological injury, the suppression of the inflammatory cytokine IL-6 and the promotion of the immunosuppressive cytokine IL-10. Previous data showed a rapid elevation of IL-6 level after reperfusion and the association to the severity of reperfusion injury in the context of PGD (25). These data are corroborated by others, e.g. Casanova et al. reported the efficacy of Sevo preconditioning in an auto-transplant pig model showing a decrease of oxidative stress marker, pro-inflammatory mediators, chemokines and adhesion molecules (9, 15). Also, a study of Rancan et al. also in lung auto-transplant in pigs showed decreased inflammatory cytokine and chemokine in liver (26).

While there is some evidence of an amelioration of PGD in syngeneic lung Tx, no reports are available so far on allo-Tx in the context of Sevo preconditioning. We showed that Sevo preconditioning attenuates AR damage histologically and upregulates M2 macrophages, cells that bear anti-

inflammatory activity. Evidence suggests that 5' adenosine monophosphate-activated protein kinase (AMPK) plays an important role in M2 macrophage polarization (27, 28). AMPK, an important sensor and regulator of cellular energy status, is activated in response to ischemic stress and is implicated in ischemic preconditioning (29). Specifically this activation of AMPK can also be induced by Sevo as shown in a sevoflurane-induced cardioprotection model (30). These data could be a hint to how alternative macrophages can act to protect the transplant through Sevo.

Shortcomings of this study are obvious: functional data of oxygenation obtained here were heterogeneous among types of experiments. This can be explained by the blood sampling procedure. We chose for taking whole arterial blood samples via the abdominal descending aorta without clamping the right hilum due to sudden death by the animal when occluding the right lung and only evaluating the left lungs oxygenation. We consider this as an indirect evaluation of the functionality of the Tx lung, however, it shows a trend towards an improvement of functionality. Only in the Allo-Tx group, lung injury on the left side in the control group could have been compensated by the right lung ventilation. Another limitation of this study was cardio-dynamic differences between volatile anesthetics and injection anesthetics of fentanyl, midazolam and medetomidine that we used for controls (31, 32). Although the iv.-narcosis is widely used in the clinical setting and qualifies well for this control group (equivalent to ip.-narcosis), it limits the comparability to a clinical setting due to interference with inflammatory parameters analyzed.

Sevo is already routinely used in many centers not only in North America but also worldwide for the recipient lung Tx procedure, however, there is no systematic analysis available on a beneficial effect of Sevo on lung transplant outcome in these recipients. Also, Sevo preconditioning in donor lung organs has not been performed yet in order to analyze an additional beneficial outcome in lung transplanted patients. Some evidence towards a positive effect of Sevo preconditioning in human liver transplants exists, but overall evidence remains scarce.

Moreover, studies available on transplant outcome and Sevo preconditioning are limited to the analysis of the impact on PGD, but there is no experimental work investigating AR. We could therefore provide here the first evidence of a beneficial effect of Sevo donor organ preconditioning on post lung transplant outcome in PGD and AR.

Clinically, Sevo preconditioning in a pre-transplant setting would be well doable without much changing the pre-operative set up. Also, a suitable possibility for preconditioning would be the use of the ex vivo lung perfusion system (1). If Sevo preconditioning positively influences lung transplant outcome, this measure would be a valuable addition to Sevo-narcosis in lung transplant recipients.

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Figure legends

Figure 1

(A) Blood gas analyses were measured on a donor graft after 2 hours of Sevo preconditioning. PaO_2/FiO_2 ratio in the Sevo organs were significantly higher than in controls (p=0.030). (B) RT-PCR on lung homogenate samples showed a relative suppression of TNF-α in the Sevo group (p=0.20). Asterisk indicates significant differences.

Figure 2

Representative HE pathology of syngeneic lung Tx on day 1: (A) Grafts in the control group showed a typical pathology of PGD with diffuse septal thickening, edema and cell infiltrations. (B) Graft in the Sevo group showed attenuated PGD pathology when compared to controls.

Figure 3

Cytokines in Syn-Tx were measured by ELISA. (A) Levels of IL-6 in plasma in the Sevo group were significantly lower than in controls (p=0.012). (B) There was a tendency towards a suppression of IL-6 in lung in the Sevo group (p=0.15). (C) Also, there was a tendency towards an enhancment of IL-10 in plasma in the Sevo group (p=0.15). (D) Levels of IL-10 in lungs in the Sevo group were significantly higher than in controls (p=0.002). Asterisk indicates significant differences.

Figure 4

Representative H&E pathology of Allo-Tx recipients on day 5: (A) Grafts in controls showed a typical pathology of acute rejection with perivascular mononuclear infiltration. (B) Grafts in the Sevo group showed an attenuation of AR compared to controls (C) The rejection score showed a significant difference between the two groups (p=0.030). Asterisk indicates significant differences.

Figure 5

Representative immunohistochemical analyses of Allo-Tx mice on day 5: F4/80 staining (total macrophages) (A, B) and CD206 staining (anti-inflammatory M2 macrophage) (D, E) of the control

(A, D) and the Sevo group (B, E). There were less F4/80 positive cells in controls compared to the Sevo group (p=0.001) (C), while more CD206 positive cells were detected (p=0.003) (D). Asterisk indicates significant differences.

Figure 6

PaO_2/FiO_2 ratio in the Allo-Tx on day 5 had a tendency toward the improvement in the sevo group compared to controls (p=0.16).

Figure
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Figure 1

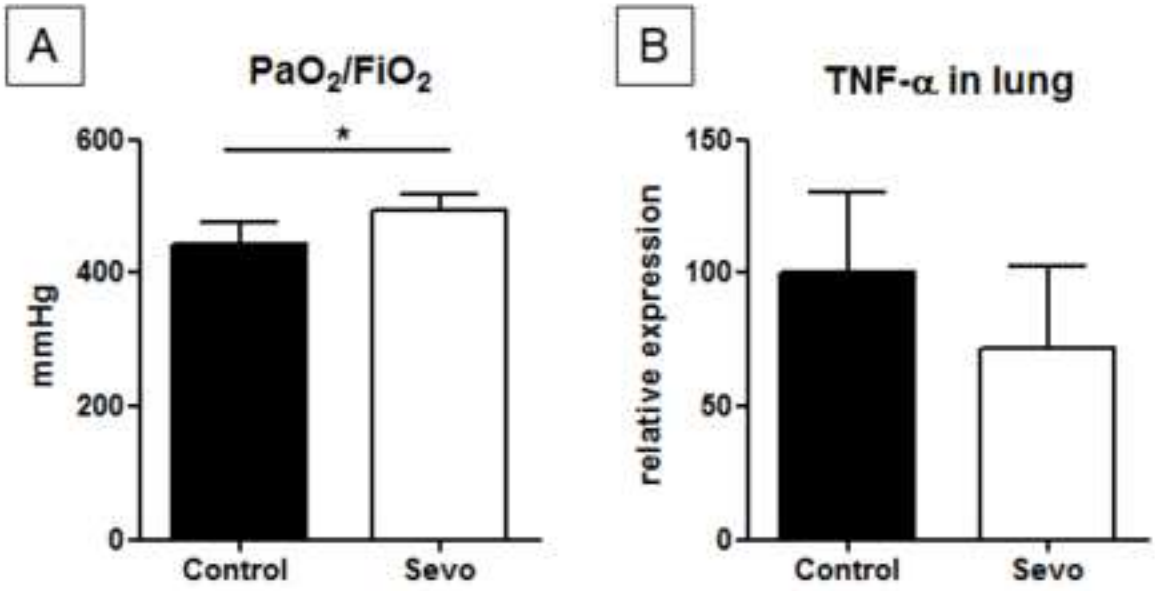

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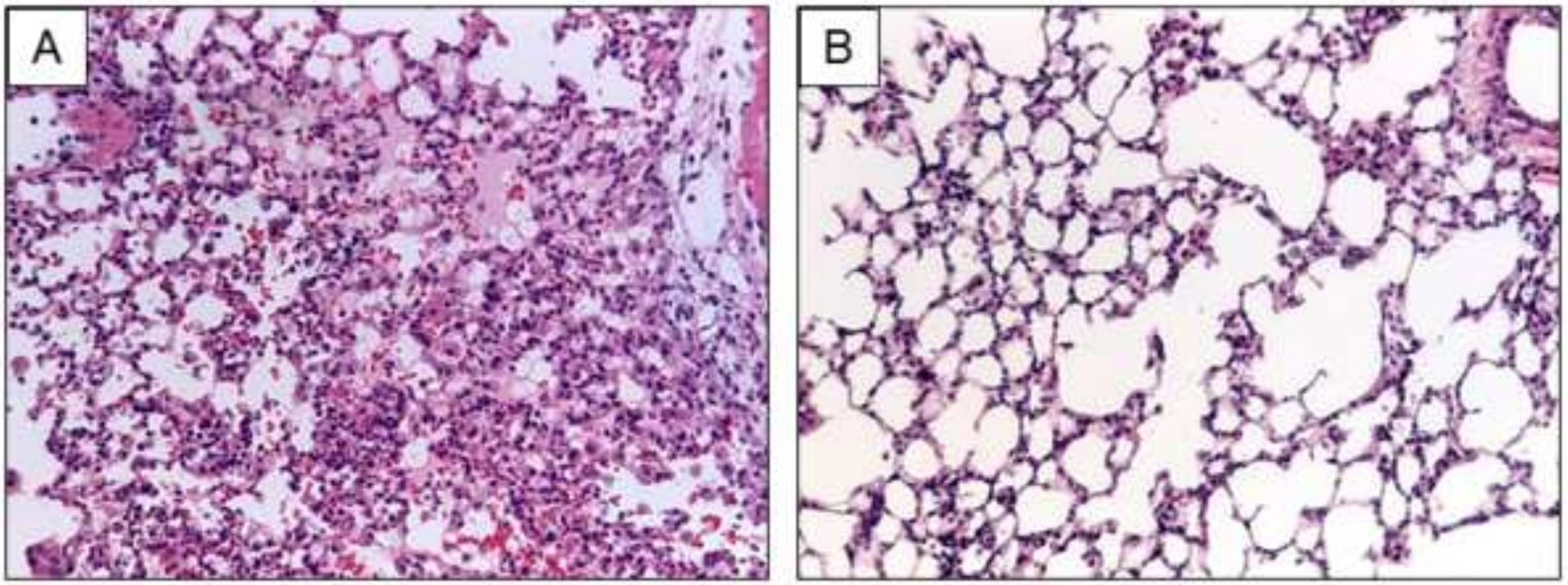
Figure 2



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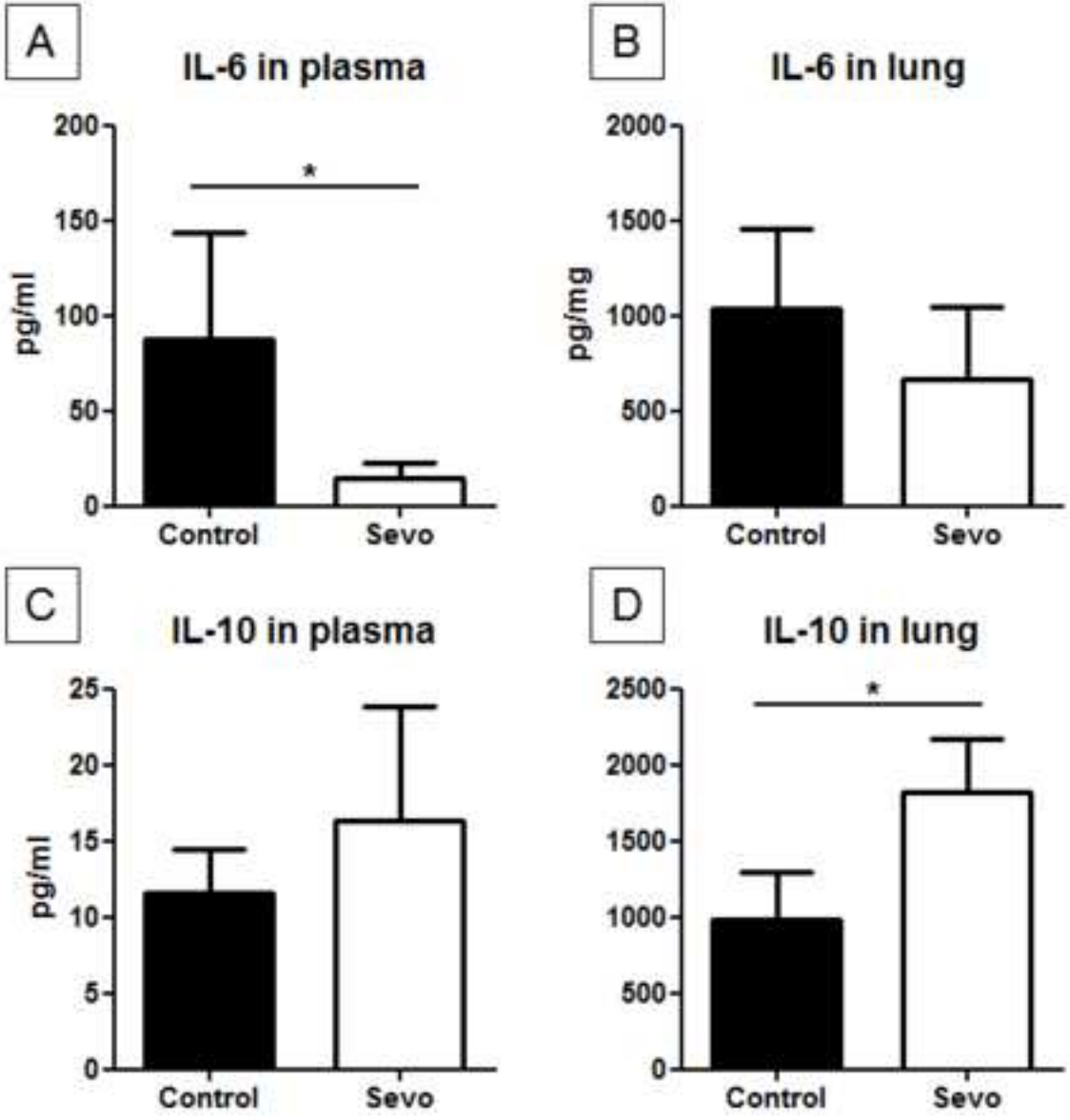

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Figure 4

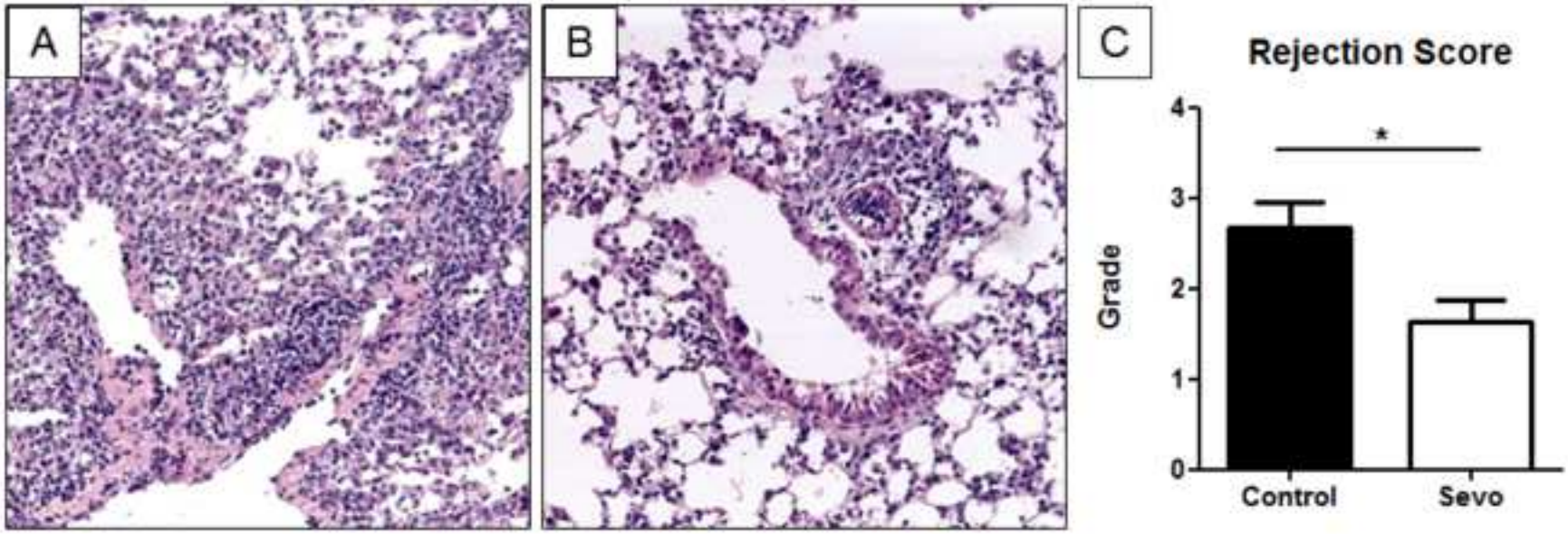

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Figure 5

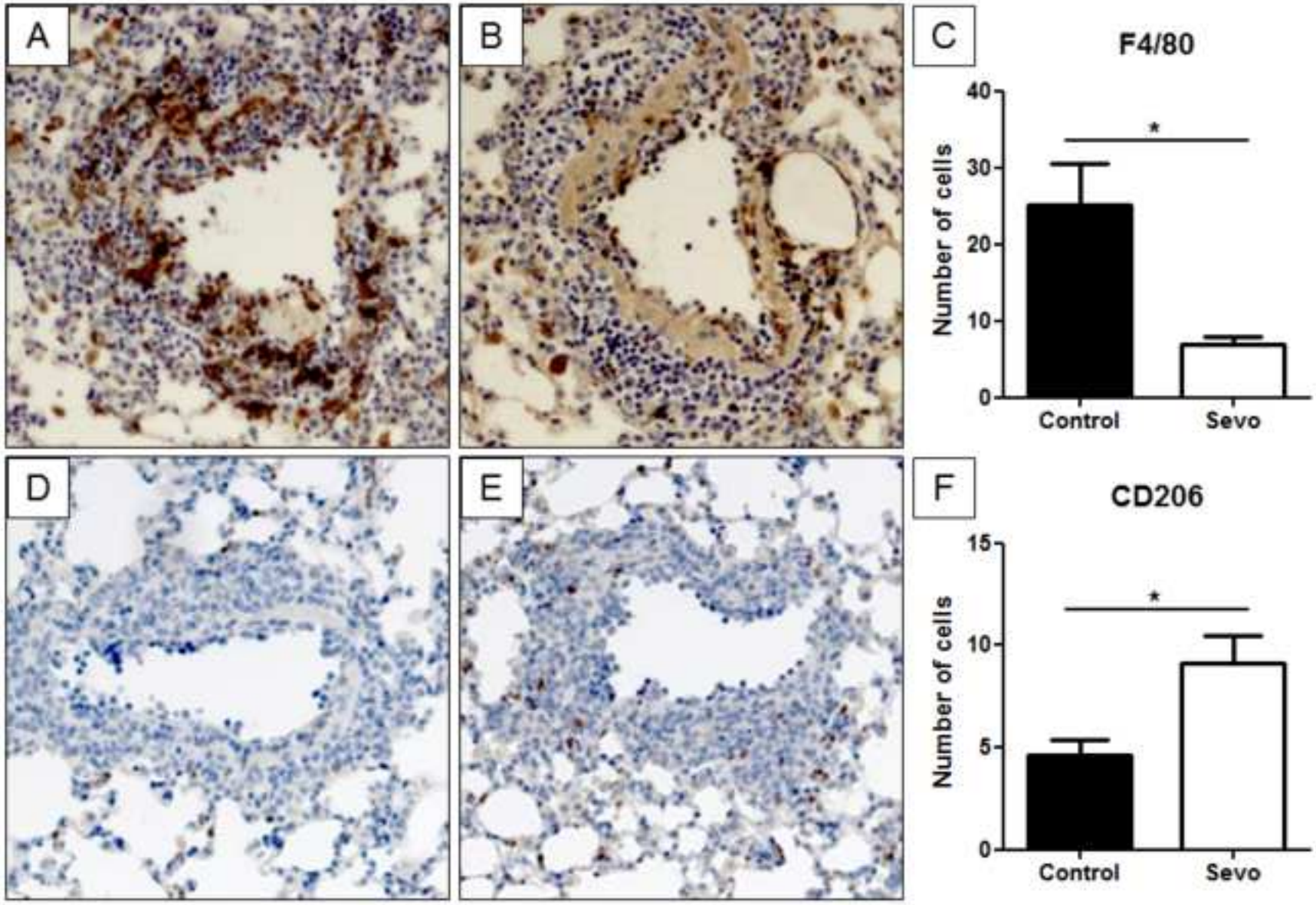

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Figure 6